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Aspects of the Testicular Toxicity of Phthalate Esters

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Di(2-ethylhexyl) phthalate (DEHP) produced seminiferous tubular atrophy and reductions in seminal vesicle and prostate weight in 4-week-old, but not in 15-week-old rats. Di-*n*-pentyl phthalate (DPP) did produce atrophy in the older rats but this developed more slowly than in young animals. Coadministration of testosterone or gonadotrophins did not protect against phthalate-induced testicular toxicity but did partly reverse the depression of seminal vesicle and prostate weight. Secretion of seminiferous tubule fluid and androgen binding protein by the Sertoli cells was markedly suppressed within 1 hr of a dose of DPP or mono-2-ethylhexyl phthalate (MEHP) in immature rats. This occurred less rapidly in mature rats. [^{14}C]Mono-*n*-pentyl phthalate and [^{14}C]MEHP penetrated the blood testis barrier only to a very limited extent. These findings and the early morphological changes in the Sertoli cells produced by DPP suggest that phthalate esters may act initially to cause Sertoli cell injury, the subsequent loss of germ cells occurring as a consequence of this.

Some features of the testicular lesion could be reproduced in primary cocultures of rat Sertoli and germ cells. Structure activity studies with a range of phthalate monoesters showed good agreement between the induction of germ cell detachment in culture and testicular toxicity *in vivo*. Three metabolites of MEHP (metabolites V, VI, and IX) were much less toxic in culture than MEHP itself, suggesting that the latter may be the active testicular toxin from DEHP.

Introduction

The testicular toxicity of di(2-ethylhexyl) phthalate (DEHP) has been recognized for many years (*1–3*). In rats, repeated administration of DEHP results in seminiferous tubular atrophy characterized by a loss of the meiotic and post-meiotic germ cell populations from the seminiferous epithelium (*4*). Certain other esters, such as di-*n*-butyl-, di-*n*-pentyl-,and di-*n*-hexyl phthalate, produce the same lesion whereas others, such as diethyl and di-*tert*-butyl phthalate, have no effect on the testis (*5–6*). Phthalate diesters are initially metabolized to the corresponding monoester and alcohol moieties. The testicular lesion can be reproduced by administration of the monoester but not by the alcohol (*7–8*). Whether further metabolism of the monoester (*9*) is required has not yet been established.

Mice, guinea pigs, and ferrets are also susceptible to the induction of testicular injury by phthalate esters, but Syrian hamsters are comparatively resistant (*8,10*). The lesion is similar in all susceptible species, involving early detachment of spermatocytes and spermatids from the seminiferous epithelium, but little is known of the mechanisms underlying this effect. However, Creasy et al. (*4*) reported that morphological changes were evident in the Sertoli cells within 3 to 6 hr of a single dose of di-*n*-pentyl phthalate, at which time the germ cell population appeared unaffected.

This paper describes some features of the testicular effects of phthalate esters in rats, with emphasis on the Sertoli cell as a primary site of action and on the effects of phthalate monoesters on testicular cell cultures. A review of the biological effects of phthalate esters, including their testicular toxicity has recently been published (*11*).

Experimental

Materials

DEHP, dibutyl phthalate (DBP), and diethyl phthalate were obtained from BP Chemicals Ltd., South Glamorgan, U.K., and di-*n*-pentyl phthalate from Eastman Kodak. Phthalate monoesters were synthesized by the method of Albro et al. (*12*). Testosterone propionate was obtained from Sigma Chemical Company and pregnant mares' serum gonadotrophin (PMSG) from Paines and Byrne Pharmaceuticals, Greenford, U.K. 5 α-Dihydro[1α,2α (*n*)-^{3}H]testosterone, 54 Ci/mmole, was purchased from Amersham International (Amersham, U.K.). All cell culture materials were from sources cited previously (*13*).

Methods

Male Sprague-Dawley rats (Olac 1976 Ltd., Bicester, U.K.) were used at 4 to 5 weeks of age unless otherwise

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indicated. Phthalate esters were administered orally as corn oil solutions at the dose levels and for the durations indicated in the text. In one study, phthalate monoesters were given intravenously as aqueous solutions in ammonium chloride. Testosterone propionate and PMSG were administered by subcutaneous injection as corn oil and aqueous solutions respectively.

For studies on Sertoli cell function, the efferent ducts of the right testis were ligated under barbiturate anesthesia 1 hr after phthalate administration. Testis weight increases linearly over the first 24 hr following efferent duct ligation (EDL), indicating a constant rate of seminiferous tubule fluid secretion by the Sertoli cells (*14*). Androgen-binding protein (ABP) also accumulates at a constant rate for at least 16 hr after EDL (*15*). Therefore the increases in weight and ABP concentration in the ligated testis compared with the contralateral unligated testis after a 16-hr period were used as indices of fluid and ABP production, respectively. ABP was measured by the charcoal-dextran procedure of Schmidt et al. (*16*).

Passage across the blood–testis barrier was evaluated by intravenous administration of radioactively labeled compounds to rats which had undergone bilateral EDL 16 hr earlier. At 25 min after administration, samples of fluid from the rete testis and blood from the abdominal aorta were collected. The ratios of radioactivity in the rete testis fluid to that in the plasma was used as an index of penetration across the blood-testis barrier (*17*). [^{14}C]Phthalate monoesters were diluted with unlabeled monoester for administration at a dose level of 30 mg/kg.

Primary mixed cultures of Sertoli and germ cells were isolated, cultured, and treated as described previously (*13*). Studies with the following metabolites of mono-2-ethylhexyl phthalate were undertaken: mono-(5-carboxy-2-ethylpentyl) phthalate, metabolite V according to Albro et al. (*9*), mono(2-ethyl-5-oxohexyl) phthalate, metabolite VI, and mono(2-ethyl-5-hydroxyhexyl) phthalate, metabolite IX. In these experiments an incubation temperature of 32°C was used, and 1 mM sodium pyruvate was included in the culture medium (*18*).

Results

Effects of Age and Hormones on Induction of Testicular Atrophy

Administration of DEHP to 4-week-old rats at 2800 mg/kg/day for 10 days produced a marked depression in the weight of the testes, seminal vesicle, and prostate (Table 1). When 10-week-old rats were treated in the same manner, there was only a slight reduction in testis weight but the weights of the seminal vesicle and prostate were significantly reduced. In 15-week-old rats, DEHP had no effect on any of these organ weights. Histologically, the testes of the 4-week-old rats showed severe atrophy affecting virtually all tubules. These were populated only by Sertoli cells, spermatogonia, and occasional primary spermatocytes. In the 10-week-old rats, these histological changes were evident in 5 to 50% of tubules, the remainder appearing essentially normal. No histological abnormalities where seen in testes from the 15-week-old rats. However, di-*n*-pentyl phthalate (2200 mg/kg/day) did produce tubular atrophy in 15-week-old rats. Although changes were evident within 24 hr, the lesion was initially less severe and developed more slowly than in immature animals.

The possibility that phthalate esters induce testicular atrophy by interfering with the production of testosterone or the pituitary gonadotrophins was examined in studies involving co-administration of these hormones with di-*n*-butyl phthalate (DBP). Rats treated with DBP, 2000 mg/kg/day for 5 days, showed a significant reduction in testis and seminal vesicle weight (Table 2) and severe testicular atrophy. Administration of 50 units of pregnant mares' serum gonadotrophin (PMSG) on the first 2 days of DBP treatment did not influence the effects of DBP on the testis. However, the weight of the seminal vesicle was increased markedly after PMSG treatment in both the control and DBP-treated rats (Table 2). Very similar results were obtained when testosterone propionate (200 μg/kg/day) was administered daily during treatment with DBP. Development of the testicular lesion was not affected but there was a 30 to 50% increase in seminal vesicle weight in the control and treated groups.

Effects on Sertoli Cell Function

The observations described above suggested that the testicular lesion was not primarily due to lack of availability of pituitary hormones or testosterone, thus pointing to a site of action in the seminiferous tubules. In view of the early morphological changes observed in the Sertoli cells (*4*), we studied the effects of some phthalate esters on two specific markers of Sertoli cell function, the secretion of seminiferous tubule fluid and of androgen binding protein (ABP). Table 3 shows that after a single dose of 2200 mg/kg di-*n*-pentyl phthalate (DPP), production of fluid and ABP was almost completely suppressed. This effect was still marked at a dose level of 440 mg/kg but was not evident at 220 mg/kg. After three daily doses of DPP at 220 mg/kg, one out of five rats was partially affected. Mono-2-ethylhexyl phthalate (MEHP), at a dose level of 1000 mg/kg, reduced fluid and ABP production to around 50% of control after a single dose, and 25% of control after three daily doses (Table 3). In contrast, diethyl phthalate, an ester which does not cause testicular atrophy (*5*), had no effect on these criteria of Sertoli cell function after three daily doses of 1600 mg/kg, a dose level equimolar with 2200 mg/kg of DPP (Table 3).

When 10-week-old rats were given a single dose of DPP, at 2200 mg/kg, fluid and ABP production were only reduced to around 60% of control, while MEHP, 1000 mg/kg, produced no effect (Table 4). MEHP was still without effect after three daily doses, whereas DPP resulted in marked suppression of both fluid and ABP production.

A limited study on the passage of phthalate monoesters across the blood–testis barrier was conducted by comparing concentrations in the rete testis fluid and plasma

Table 1. Age-dependent effects of di(2-ethylhexyl) phthalate (DEHP) on male reproductive organ weights in rats.[a]

Age, weeks	Treatment	Testes, mg	Seminal vesicle, mg	Prostate, mg	Body, g
4	Control	1380 ± 40	54 ± 4	88 ± 5	136 ± 4
	DEHP	720 ± 50*	27 ± 2*	46 ± 2*	106 ± 4*
10	Control	2650 ± 110	1328 ± 42	358 ± 23	300 ± 6
	DEHP	2230 ± 180	678 ± 85*	220 ± 18*	238 ± 11*
15	Control	3330 ± 94	1625 ± 89	500 ± 41	399 ± 6
	DEHP	3170 ± 70	1480 ± 60	453 ± 27	354 ± 7*

[a] Groups of 8 rats of the indicated age at the start of treatment were given DEHP, 2800 mg/kg/day orally for 10 days. Values are means ± SEM.
*Significantly different from control, $p < 0.001$ (Student's t-test).

Table 2. Effect of pregnant mares' serum gonadotrophin (PMSG) on di-*n*-butyl phthalate (DBP)-induced changes in male reproductive organ weights in immature rats.[a]

	PMSG	Control	DBP	% Control
Testes, mg	−	952 ± 29	629 ± 37*	66
	+	859 ± 49	564 ± 31*	66
Seminal vesicle, mg	−	38 ± 2	24 ± 3†	63
	+	115 ± 4	91 ± 10‡	79
Body weight, g	−	104 ± 4	99 ± 2	95
	+	100 ± 2	98 ± 2	98

[a] Groups of 6 rats were given DBP, 2000 mg/kg/day orally for 5 days; 50 units of PMSG administered subcutaneously on the first 2 days of DBP treatment. Values are mean ± SEM.
*Significantly different from corresponding control, $p < 0.001$ (Student's t-test).
†Significantly different from corresponding control, $p < 0.01$ (Student's t-test).
‡Significantly different from corresponding control, $p < 0.05$ (Student's t-test).

Table 3. Effect of di-*n*-pentyl phthalate (DPP), mono-2-ethylhexyl phthalate (MEHP), or diethyl phthalate (DEP) on secretion of seminiferous tubule fluid and androgen-binding protein (ABP) in immature rats.

		Single dose		Three daily doses	
Treatment[a]	Dose, mg/kg	Fluid secretion, mg/testis[b]	ABP production, pmole/testis[b]	Fluid secretion, mg/testis	ABP production, pmole/testis
Control	0	166 ± 15	21.9 ± 1.2	186 ± 17	16.9 ± 0.9
DPP	220	154 ± 7	21.1 ± 1.1	164 ± 46	14.6 ± 2.9
DPP	440	44 ± 24†	5.78 ± 3.87†	—	—
DPP	2200	3 ± 3*	0.00	—	—
MEHP	1000	87 ± 20‡	12.1 ± 3.5	52 ± 19*	3.76 ± 2.01*
DEP	1600	—	—	172 ± 25	15.2 ± 2.5

[a] Compounds were administered orally, and unilateral efferent duct ligation was carried out 1 hr after the last treatment.
[b] Values represent total production over a 16-hr ligation period and are means ± SEM for groups of five rats.
*Significantly different from control, $p < 0.001$ (Student's t-test).
†Significantly different from control, $p < 0.01$ (Student's t-test).
‡Significantly different from control, $p < 0.05$ (Student's t-test).

Table 4. Effect of di-*n*-pentyl phthalate (DPP) or mono-2-ethylhexyl phthalate (MEHP) on secretion of seminiferous tubule fluid and androgen-binding protein (ABP) in 10-week-old rats.

		Single dose			Three daily doses		
Treatment	Dose, mg/kg	Fluid production, mg/testis[a]	ABP production, pmole/testis	Weight unligated testis, g	Fluid production, mg/testis	ABP production, pmole/testis	Weight unligated testis, g
Control	0	456 ± 44	33.9 ± 2.9	1.48 ± 0.08	414 ± 15	28.5 ± 2.3	1.46 ± 0.07
DPP	2200	296 ± 60	19.6 ± 3.8‡	1.41 ± 0.05	70 ± 7*	1.24 ± 1.02*	1.12 ± 0.02†
MEHP	1000	432 ± 41	32.3 ± 2.8	1.47 ± 0.06	448 ± 19	30.9 ± 1.4	1.45 ± 0.04

[a] Values represent total production over a 16-hr ligation period starting 1 hr after treatment. Means ± SEM for groups of five rats.
*Significantly different from control, $p < 0.001$ (Student's t-test).
†Significantly different from control, $p < 0.01$ (Student's t-test).
‡Significantly different from control, $p < 0.05$ (Student's t-test).

Table 5. Fraction of plasma radioactivity present in rete testis fluid after intravenous administration of ^{3}H-water and ^{14}C-phthalate monoesters to immature rats.

	Radioactivity in rete testis fluid as % of plasma radioactivity[a]
^{3}H-Water	98.7 ± 4.0
^{14}C-Mono-*n*-pentyl phthalate	1.6, 4.8
^{14}C-Mono-2-ethylhexyl phthalate	3.2, 3.3

[a] Measurements made 25 min after injection of radioactive compound, as described in methods section. Values are the mean ± SEM for three rats (^{3}H-water) or individual values from two rats.

after intravenous injection of either [^{14}C]mono-2-ethylhexyl phthalate or [^{14}C]mono-*n*-pentyl phthalate. [^{3}H]water was included as a substance known to pass readily across the barrier (*17*). Measurements 25 min after administration showed that the concentration of [^{3}H]water in the rete testis fluid was 99% of the plasma concentration whereas for both monoesters, less than 5% of the plasma level was found in the rete testis fluid (Table 5).

Effects on Testicular Cell Cultures

In view of the effects of phthalate esters on Sertoli cells and the early separation of germ cells from the Sertoli cells observed *in vivo*, we examined the use of primary cocultures of Sertoli and germ cells as an *in vitro* model for phthalate-induced testicular toxicity (*13*). The normal appearance of these cultures is shown in Figure 1. Addition of 100 μM MEHP to the culture medium for 24 hr resulted in a pronounced detachment of germ cells from the Sertoli cell monolayer (Fig. 2) and a change in Sertoli cell morphology to a more elongated shape. No such changes were produced by DEHP or its other primary metabolite, 2-ethylhexanol. By counting the numbers of germ cells detaching from the cultures, the effect of MEHP was shown to be concentration-dependent over the range of 1 to 100 μM (Table 6). In studies with a range of other phthalate monoesters, it was found that only those causing testicular damage *in vivo* produced an increase in germ cell detachment at low concentrations (1 to 100 μM) in culture (Table 6). Esters such as monoethyl phthalate produced effects only at much higher concentrations (10,000 μM).

In view of the apparent specificity of this culture system, some preliminary studies were carried out with three metabolites of MEHP, compounds V, VI, and IX in the metabolic scheme described by Albro et al. (*9*). None of these metabolites had any effect on the cultures at a concentration of 100 μM, at which level MEHP itself produced marked germ cell detachment (Table 7). Metabolites V and VI had no effect at 1000 μM, but metabolite IX did produce an increase in cell detachment. However, this concentration of MEHP was markedly toxic, causing almost complete destruction of the cultures within 24 hr. These studies with the metabolites were carried out at an incubation temperature of 32°C. At this temperature, which favors prolonged survival of the cultures, the rate of germ cell detachment is somewhat lower than at 37°C (compare data for MEHP in Tables 6 and 7).

The order of toxicity of the monoesters studied *in vitro* (Table 6) showed some differences from the findings of Foster et al. (*5*) for the corresponding diesters *in vivo*. Foster et al. (*5*) found that DPP was the most potent of the series and that di-*n*-octyl phthalate had no effect on the testis. In contrast, MEHP was the most potent ester *in vitro* and mono-*n*-octyl phthalate was also active. To determine whether these apparent discrepancies might reflect differences in rate and extent of intestinal hydrolysis and absorption *in vivo*, MEHP, mono-*n*-octyl and mono-*n*-pentyl phthalate were administered intravenously at equimolar dose levels daily for 4 days. Under these conditions, both MEHP and mono-*n*-octyl phthalate produced histological evidence of testicular damage at 30 mg/kg. In both cases, the histological changes were slight but characteristic of the phthalate lesion. At an equimolar dose level, 25 mg/kg, mono-*n*-pentyl phthalate produced no such changes, but did so at the higher level of 50 mg/kg. Higher dose levels of MEHP and mono-*n*-octyl phthalate were not well tolerated by the rats.

Discussion

Phthalate esters that cause testicular damage appear to exert their toxicity primarily on the seminiferous tubules. The studies involving supplementation with testosterone or gonadotrophins provided no evidence that deficiency of these hormones has a causal role in the phthalate-induced testicular lesion, while a role for induced nutritional or vascular disturbance is unlikely in view of the rapid onset of the damage. Histological abnormalities develop in the tubules within a few hours of phthalate administration (*4*), whereas changes in the Leydig cells and in the pituitary develop only after repeated exposure (*2,4*).

The secretion of two Sertoli cell products, seminiferous tubule fluid and androgen binding protein (*19,20*), was markedly inhibited after a single oral dose of DPP and MEHP in immature rats. This was found to occur even when measurements were started as early as 1 hr after treatment. However, since morphological changes in the Sertoli cells have been observed at 3 hr after a single dose of DPP (4), it might be anticipated that functional impairment would be evident at an earlier stage. It is generally accepted that the Sertoli cells play a key role in controlling the development and maintenance of spermatogenesis and that they constitute the main functional component of the blood–testis barrier (*20–23*). Since derangement of Sertoli cell function occurs at such an early stage after phthalate treatment, and since the germ cells affected initially are those inside the Sertoli cell barrier (and therefore most critically dependent on normal Sertoli cell function), it is reasonable to propose that the Sertoli cells are the primary target of phthalates that cause testicular injury. Loss of germ cells leading to tubular atrophy would follow as a consequence of this. A primary action on the Sertoli cells is also compatible with the observations that MEHP and mono-*n*-pentyl phthalate

Table 6. Effect of some phthalate monoesters on germ cell detachment in rat testicular cell cultures.[a]

Phthalate monoester	Testicular toxicity *in vivo*[b]	No. of germ cells detached (as % of control) at various monoester concentrations					
		1 μM	10 μM	100 μM	1000 μM	3000 μM	10000 μM
2-Ethylhexyl	+	208*	213*	384*	c	c	c
n-Octyl	+	130‡	161‡	500*	c	c	c
n-Hexyl	+	132†	199*	231*	c	c	c
n-Pentyl	+	111	147	206*	276*	c	c
n-Butyl	+	c	113	143†	165†	228*	c
Tert-butyl	−	c	c	c	115	110	307*
n-Propyl	−	c	c	c	100	99	259*
Ethyl	−	c	c	c	86	81	210*
Methyl	−	c	c	c	103	86	48*

[a] Values are means for four culture dishes after a 24-hr treatment period and expressed as a percentage of the number of germ cells detaching from corresponding control cultures (usually about 2 × 10^5 cells/24hr). Data from Gray and Beamand (*13*).

[b] Data of Foster et al. (*5,6*, and unpublished data).

‡Significantly different from control, $p < 0.05$ (Student's *t*-test).

†Significantly different from control, $p < 0.01$ (Student's *t*-test).

*Significantly different from control, $p < 0.001$ (Student's *t*-test).

[c] Not determined.

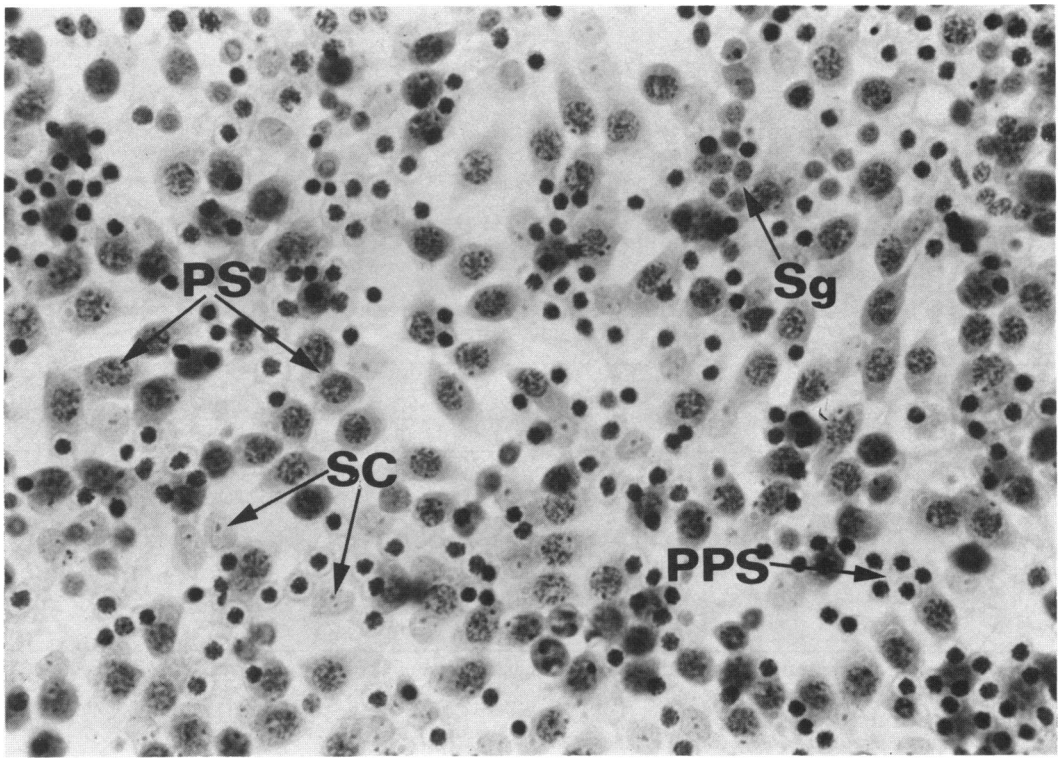

FIGURE 1. Untreated primary culture of rat Sertoli and germ cells. Numerous germ cells, comprising pachytene spermatocytes (PS), prepachytene spermatocytes (PPS) and spermatogonia (Sg), are attached to the Sertoli cell (SC) monolayer. Original magnification × 200.

did not readily cross the blood–testis barrier. A direct effect on the germ cells inside the barrier is not easy to reconcile with this finding.

Oishi and Hiraga (*24*) reported a decrease in circulating testosterone in rats administered DEHP for 5 days, and Foster et al. (*25*) found that a single dose of DPP caused a marked inhibition of 17α-hydroxylase and 17, 20-lyase activities in whole testis microsomes. These observations may help explain the reduced weights of the androgen-dependent seminal vesicle and prostate gland in phthalate-treated rats, since exogenous testosterone, or stimulation of endogenous testosterone production by administration of PMSG, increased these weights. However, in neither case were these weights restored to those in the hormone stimulated control rats, suggesting a possible effect on androgen-tissue receptor interactions.

Production of testosterone by the Leydig cells is partly controlled by factors secreted by the Sertoli cells (*26*). In view of this, changes in testosterone production following phthalate treatment could be a secondary consequence of primary Sertoli cell injury. That the Leydig cells remain capable of responding to gonadotrophin stim-

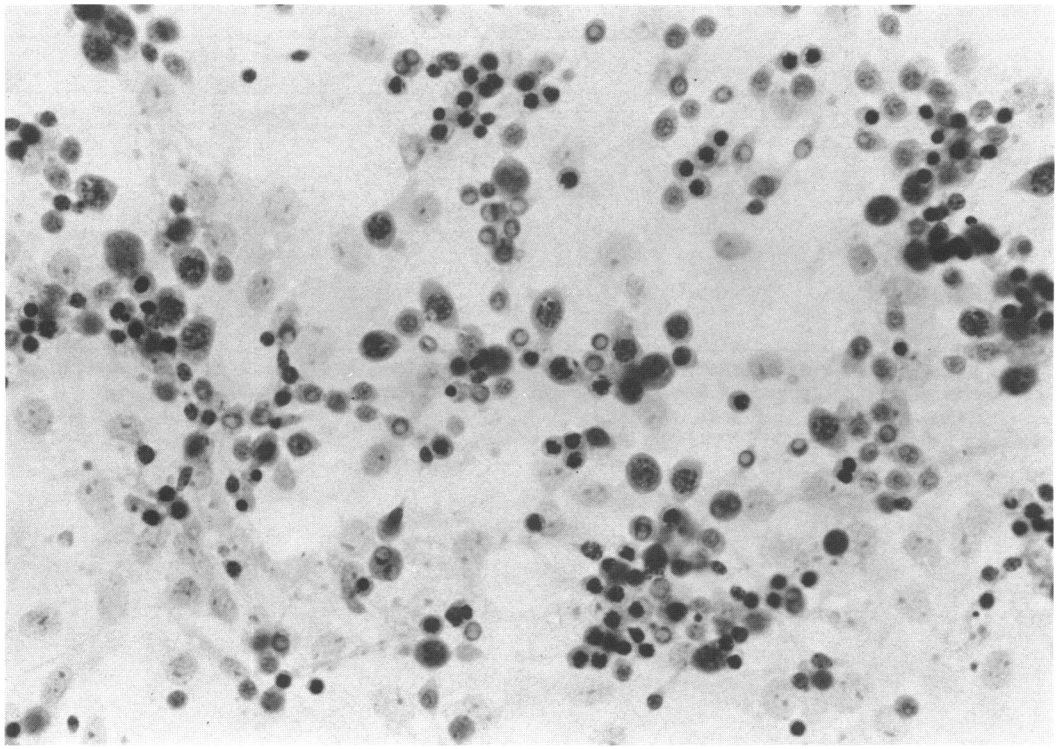

FIGURE 2. Primary culture of rat Sertoli and germ cells treated for 24 hr with 100 μM mono-2-ethylhexyl phthalate. Note loss of germ cells compared to the corresponding control culture in Fig. 1. Original magnification × 200.

Table 7. Effect of mono-2-ethylhexyl phthalate (MEHP) and three metabolites of MEHP on germ cell detachment in rat testicular cell cultures.[a]

Treatment	Treatment time, hr	No. of germ cells detached (as % of control) at various MEHP concentrations		
		10μM	100μM	1000μM
MEHP	24	141†	210†	Toxic
	48	201†	231*	Toxic
Metabolite V[b]	24	94	81	94
	48	107	100	113
Metabolite VI[b]	24	66	118	90
	48	76	115	103
Metabolite IX[b]	24	89	81	144‡
	48	104	104	224†

[a] Values are means for four or five culture dishes from one experiment typical of at least three, and are expressed as a percentage of the number of germ cells detaching from corresponding control cultures.
[b] For definition, see "Methods" section.
*Significantly different from control, $p < 0.001$ (Student's t-test).
†Significantly different from control, $p < 0.01$ (Student's t-test).
‡Significantly different from control, $p < 0.05$ (Student's t-test).

ulation after phthalate exposure is indicated by the stimulatory action of PMSG on seminal vesicle and prostate weights in DBP-treated rats.

The basis of the age-related changes in the susceptibility of the testis to phthalate esters is not clear. Alterations in Sertoli cell functions occur during sexual maturation, e.g., in response to FSH stimulation (*22,27*), but this would not explain the observed difference between 10- and 15-week-old rats in their response to DEHP. In addition to physiological changes in the testis, other factors such as age-related differences in absorption, metabolism, and distribution are probably involved, particularly since DPP did affect the 15-week-old rat. Further work is needed to resolve these issues.

The rapid shedding of germ cells produced by phthalates in the intact testis, was mimicked by the accelerated detachment of germ cells from the testicular cell cultures treated with phthalate monoesters. Germ cells detaching from the Sertoli cell monolayer were viable and morphologically normal but there were changes in Sertoli cell morphology (*13*). Thus it is possible that the action of phthalates *in vitro*, as *in vivo*, may be mediated via a

primary effect on the Sertoli cells. The generally good agreement between testicular toxicity *in vivo* and production of germ cell detachment *in vitro* suggests that this culture system may be useful both for screening other phthalate esters and also for studying the mechanism of their toxicity.

The observations in cell culture point to the monoester, rather than the alcohol or intact diester, as the mediator of the testicular lesion. MEHP caused germ cell detachment *in vitro* at concentrations as low as 1 μM whereas the three metabolites studied here were comparatively nontoxic at 100 to 1000 times this concentration. These metabolites comprise 67% of the urinary total in rats after a dose of DEHP (*9*). Thus, MEHP itself may be the active testicular toxin. However, more extensive studies with these and other metabolites *in vivo* and *in vitro* will be necessary to clarify this issue.

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